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RESEARCH ARTICLE

The juvenile hormone analogue, pyriproxifen, alters protein and fat composition of *Tenebrio molitor* larvae

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Abstract

Maximising the yield of product from livestock is common practice in the agriculture industry and there is potential to extend this practice to the emerging insect industry, to produce high-quality, sustainable protein. *Tenebrio molitor* larvae, commonly called yellow mealworms, were fed for 28 days on wheat bran containing the juvenile hormone analogue, pyriproxifen at either 2 mg pyriproxifen/kg wheat bran (JH-PL) or 15 mg pyriproxifen/kg wheat bran (JH-PH). As expected, pupation was inhibited in both pyriproxifen treated groups and significant changes in nutritional composition were observed. Pyriproxifen treated mealworms had a higher protein content per 100 grams of dried material, while fat content was reduced 68% in JH-PH compared to control. These changes were associated with an increase in moisture content and reduction in energy content. The fatty acid profile of extracted fat also displayed significant alterations, with pyriproxifen treated mealworms showing an increase in proportions of saturated fatty acids, reduction in oleic acid but no effect on linoleic acid. The amino acid composition also exhibited a change in composition as a result of pyriproxifen treatment, including an increase in the essential amino acid, lysine, in JH-PH treated mealworms. This change in amino acid profile was associated with a change in the protein composition as observed on SDS-PAGE, with the appearance of a new band identified as the egg-yolk protein, vitellogenin, which has lipid-transporter activity. Hence, pyriproxifen treatment of mealworms has a repartitioning effect, resulting in an increase in the proportion of protein and a decrease in fat on a dry matter basis, demonstrating that mealworm nutrient composition can be manipulated to provide a higher value feed ingredient.

Keywords

growth – nutrient composition – *Tenebrio molitor*

1 Introduction

There is increasing concern about the sustainability of protein production for both direct human consumption and use in animal feed (Salter and Lopez-Viso, 2021). Insects have been identified as an alternative nutrition source, particularly for protein (Hawkey *et al.*, 2021; van Huis *et al.*, 2013). *Tenebrio molitor* L. larvae (yellow mealworms, referred to as mealworms) have high nutritional value (Ghaly and Alkoaik, 2009; Hawkey *et al.*, 2021), as well as the versatility to grow on different low quality substrates (Alves *et al.*, 2016; Yang *et al.*, 2019). In order to be used as a livestock feed ingredient, the focus on mealworms tends to be maximizing the protein

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yield and minimising the fat content, with the reported ranges being 46-54% protein per unit dry weight of mealworms and 25-36% fat per unit dry weight of mealworms (Hawkey *et al.*, 2021). Given the variation in these values it may be possible to improve the mealworm composition by manipulating the protein and/or lipid composition. Mealworms have a relatively long larval stage (8-10 weeks), and in insects that undergo metamorphosis it appears that the critical body weight (also known as threshold size), which is associated with the accumulation of protein and fat, is a key factor in determining the onset of metamorphosis (Malita and Rewitz, 2021; He *et al.*, 2020). The process of larval growth and the transition to metamorphosis is also regulated by a range of endocrine factors. Ecdysteroids are involved in inducing molting (Lafont *et al.*, 2012) and are important activators of metamorphosis (Cherbas *et al.*, 2003), with 20-hydroxyecdysone (20E) being the most active form. Juvenile hormones are sesquiterpenes that have a variety of functions, including a significant role in regulating progression through the different life stages during growth and metamorphosis (Jindra *et al.*, 2013). Juvenile hormone (JH) was originally discovered for its capacity to prevent metamorphosis, maintaining the larval stages (Wigglesworth, 1934). The response to exogenous JH varies between orders of insect, with Coleoptera and Lepidoptera larvae undergoing additional instars, thereby becoming giant larvae (Truman, 2019). At a nutritional level, both 20E and JH have been shown to play a role in lipid metabolism. The steroid hormone 20E has been described as activating/enhancing lipolysis and accelerating fatty acid β-oxidation in the silkworm, *Bombyx mori* L. and in *Hyphantria cunea* (Drury) larvae (Houssain *et al.*, 2013; Zhang *et al.*, 2023), whilst JH was previously described as accelerating lipid synthesis. In *Drospohila melanogaster* (Meigen), downregulating JH signalling reduces the quanity of lipid in the fat body of larvae (Liu *et al.*, 2009).

From a production animal perspective increasing animal size is a fundamental objective, but ideally this should be achieved by increasing the production efficiency (i.e. growth per unit feed), which is why the value of a feed or food ingredient is associated with the density of protein within it. In production animals used for food, such as pigs and poultry, production efficiency has been increased by selective breeding but also by the use of exogenous growth promoting agents, such as growth hormone and beta-adrenergic agonists (Brameld and Parr, 2016). Growth promoters are licenced for use in production animals in several countries in the world, but not in the European Union. However, researchers have still studied the effects of these agents in order to gain insights into the mechanisms regulating nutrient deposition in production animals (Brown *et al.*, 2018) and the consequences for product quality (Kemp *et al.*, 2010). This information has subsequently been used to identify genes and polymorphisms for molecular marker-assisted breeding selection (Zalewska *et al.*, 2021). In a similar way, application of exogenous agents known to alter insect body composition should help identify the capacity and potential limits of altering body composition and may lead to a better understanding of the mechanisms involved.

A number of JH analogues, such as methoprene and pyriproxyfen, are used as pesticides, as they tend to cause abnormal development (Parthasarathy and Palli, 2021). As a pesticide methoprene is typically used between 0.7 and 5 mg/kg in non-food cereals to restrict insect growth (Diacon II™, 2010), while pyriproxyfen tends to be applied to surfaces at 3-9 mg/m^2 to control insect infestation (Pivot10™, 2011). There are restrictions on the use of these agents for environment insect pest control or for application to plants and seeds to reduce insect infestation. For example, the minimal risk levels for pyriproxyfen recommended by the European Food Standards Agency (EFSA), is in the range 0.01-15 mg/kg (EFSA *et al.*, 2022). Hence, there is both existing regulation on the application of JH analogues, as well as the general principle of the prohibition of pharmaceutical growth promoter use in food production livestock. Both of these would likely prevent the use of JH analogues in insect food production systems. However, since JH is known to influence lipid deposition, these analogues could be used as an experimental tool to investigate the regulation of lipid (and protein) deposition in mealworms. This has not previously been fully elucidated in production related insect species. As mealworms mature in their larval stage they accumulate more lipid, which has the effect of decreasing the protein content (Lopez-Viso *et al.*, 2023; Kröncke *et al.*, 2023), and likely relates to their preparation for pupation (Li *et al.*, 2019). Our hypothesis was that the JH analogue, pyriproxyfen, would decrease fat deposition and therefore increase the proportion of protein deposited in mealworms. Also, since pupation would also be reduced, then the mealworms would continue to grow, thereby increasing their weight and yield. This is a novel approach as, to the best of our knowledge, this is the first time that an investigation has been carried out to explore the extent to which growth and nutrient deposition can be manipulated in a production related insect species utilising an exogenous endocrine factor as an experimental tool. Hence

the aim of this study was to determine the impact on larval growth (before pupation) and body composition of administering the JH analogue, pyriproxifen, to mealworms for 28 days.

2 Materials and methods

Mealworm feeding trial

Mealworms were supplied by Monkfield Nutrition Ltd (Mepal, UK) at a length of 15-18 mm. At the start of the trial, the mealworms were approximately 6 weeks old, however the exact hatch date was not provided by the suppliers. On arrival, mealworms were housed in the dark in an incubator (Weiss Technik, Loughborough, UK) at 25 °C and 60% humidity and fed *ad libitum* on organic wheat bran (Buy Whole Foods Online, Ramsgate, UK) that was standardised by sieving to a particle size between 2 and 0.5 mm. Water was supplied via a 1 cm diameter cotton wool ball soaked in deionised water which was replaced twice a week. After 3 days acclimatisation and growth, any mealworms passing through a 2 mm sieve (Retsch, Haan, Germany) were removed, with the remainder being randomly allocated to 6 × 9 × 16 cm containers (300 mealworms per container) and one of three treatment groups (n = 4 containers/treatment). Vehicle control (control) was 1.2 ml acetone added per 100 g wheat bran, while low (L) and high (H) doses of the juvenile hormone (JH) analogue, pyriproxifen (PESTANAL, Sigma Aldrich, Gillingham, UK) were made using the same volume of acetone but at concentrations that resulted in 2 mg pyriproxifen/kg wheat bran (JH-PL) or 15 mg pyriproxifen/kg wheat bran (JH-PH). These doses are within the general range of those normally applied when JH analogues are used as pesticides. The pyriproxifen solutions were first added to 10 g of wheatbran, mixed by shaking in a sealed plastic bag, then the remaining 90 g of wheat bran was added to the bag and mixed again to ensure even distribution within the wheatbran feed. The acetone was then allowed to evaporate for 1 hour at room temperature before use. The diets were fed at 10 g wheat bran per container, which, together with the water, was replaced every 3 or 4 days over the 28 day study. On measurement days, the contents of each container were emptied into a 2 mm sieve with a 0.5 mm sieve below, such that the mealworms (live, dead and pupated) remained in the top 2 mm sieve, the feed was retained by the 0.5 mm sieve, whereas the frass passed through them both. Any dead or pupated mealworms were removed and the numbers recorded, but these were not used for any further analysis. Average individual mealworms weight was calculated by dividing the group weight by the number of live mealworms remaining. At the end of the trial, after completing the above measurements, mealworms were divided into equal halves (by weight) and placed in separate plastic sealable bags: half for proximate nutrient analysis and the other half for RNA extractions. All mealworms were then culled by submersion in liquid nitrogen and stored at –80 °C.

Nutrient analysis

The samples (n = 4 per treatment group) stored at –80 °C (containing only live mealworms culled at the end of the trial, with dead or pupated mealworms being excluded) were freeze dried (Christ Freeze Dryer, Osterode am Harz, Germany), then ground and mixed to a fine powder in a liquid nitrogen cooled pestle and mortar, then stored in a desiccator for subsequent analyses.

Gross energy was determined by bomb calorimetry (6300 calorimeter, Parr Instrument Company, Moline, IL, USA) using 1 g freeze-dried sample and standards of calorific grade benzoic acid (Parr Instrument Company, Moline, IL, USA).

Total fat content was determined via Soxtherm extraction (Gerhardt Analytical Systems, Königswinter, Germany) using 1 g freeze-dried mealworms as described previously (Plamquist and Jenkins, 2003). In brief, the sample was boiled in petroleum ether at 150 °C for 30 minutes followed by refluxing for 90 minutes to ensure all fat was collected. The solvent was evaporated to dryness and the weight of fat determined. Fat samples were then resuspended in hexane and kept at –20 °C for subsequent fatty acid analysis.

To determine fatty acid contents, the triacylglycerol (TAG) was hydrolysed followed by fatty acid methyl esterification (FAME), as described by Lock *et al.* (2005), with samples stored in hexane at –20 °C before analysis using gas chromatography mass spectrometry (GCMS) as previously described (Gedi *et al.*, 2017). The system consists of the Thermo Scientific Trace 1300 gas chromatography system with helium as the carrier gas and column operating parameters of a 5 min retention time at 140 °C, then a temperature gradient of 4 °C/min up to 240 °C, then held at this temperature for 10 minutes to complete the run. A Thermo Scientific ISQ 7000 (Thermo Fisher Scientific™, Loughborough, UK) was used for mass spectrometry. The standards used were the Supelco 37 Component FAME mix (Merck, Gillingham, UK). Data outputs were analysed using Chromeleon 7 (Thermo Scientific™, Lough-

borough, UK) and proportions of individual fatty acids were calculated relative to the total amount of all fatty acids detected by GCMS.

Crude protein content was determined via the nitrogen content using an EA 1112 elemental analyser (Thermo Scientific™, Loughborough, UK) and 50 mg freeze-dried sample and aspartic acid standards (10.52 (w/w) N%, Elemental Lab, Okehampton, UK). A standard conversion factor of 6.25 was used to convert nitrogen into total protein.

Amino acid composition of freeze-dried samples was determined according to a method established in our laboratories (Muleya *et al.*, 2023). Briefly, either 30 mg (if crude protein content >60% (w/w)) or 50 mg (if crude protein content <60% (w/w)) of sample were used in order to equate to approximately 5 mg nitrogen. Every 10 samples a standard of 30 mg of Soy Flour (Standard Reference Material® 3234 Soy Flour, National Institute of Standards and Technology, Boulder, CO, USA) was analysed. Samples were first oxidised in performic acid (10% hydrogen peroxide in formic acid) at 4 °C for 18 hours followed by acid hydrolysis in 6M HCl at 110 °C for 24 hours. Hydrolysed samples were then analysed via Vanquish uHPLC and an Altis Triple Quadrapole Mass Spectrometer, with an Opta-Max NG ion source and an Acclaim™ Trinity P1 mixed mode column, controlled by TSQ Altis Tune Application XCalibration software, with data integration and extraction carried out using TraceFinder 4.1 software (Thermo Scientific™, Loughborough, UK). Amino acid proportions were calculated via the addition of all amino acids detected, excluding tryptophan which is not determined using this method.

Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (*SDS-PAGE*)

Samples (200 mg) of freeze-dried mealworm powder (the same as used for nutrient analysis) were homogenised in 1 ml of extraction buffer (150 mM NaCl, 50 mM HEPES, 2.5 mM EDTA, 10% glycerol, 1% Triton, Milli-U water), then centrifuged at 15,000 *g* and 4 °C for 10 minutes. The soluble protein content of the supernatant was determined via the Lowry assay (Lowry *et al.*, 1951) and then diluted with Lammeli loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.1 M Dithiothreitol, 10% glycerol) to adjust samples to an equal protein concentration. Constant protein was then loaded onto a 4-15% polyacrylamide gel (Criterion™ TGX Stain-Free™, Bio-Rad, Watford, UK) and separated by electrophoresis for 30 minutes. Gels were stained with Coomassie blue (GelCode™ Blue Safe Protein Stain, Thermo Scientific™, Loughborough, UK) for 3 hours then destained with deionised water. For proteomic analysis, bands of interest were extracted from the gel and sent to the York Metabolomics and Proteomics lab (Technology Facility, Department of Biology, University of York, York, UK), who carried out a trypsin digest and subsequent analyses with Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF)-tandem Mass Spectrometry, using a Bruker ultraflexTreme MALDI-TOF/TOF followed by identification using the database of *T. Molitor* sequences.

Statistical analyses

Data were analysed by one- (treatment) or two-way repeated measures (time being the repeated factor) ANOVA using Genstat statistical software (21st edition) followed by *post hoc* Bonferroni tests when appropriate. Data are presented as the means ± standard error of the mean, with significant differences accepted at $P < 0.05$.

3 Results

Effects of the juvenile hormone analogue, pyriproxifen, on mealworm growth characteristics

For average individual mealworm weight there was a trend for a time × treatment interaction ($F_{16,72} = 3.81$, $P = 0.052$), due to JH-PL tending to be bigger/ heavier from around 11 days (Figure 1A,B). As expected, there was a significant effect of time ($F_{8,72} = 259.64$, $P < 0.001$), with the mealworms increasing their body weight over the 28 days of the trial, but no significant effect of treatment ($F_{2,72} = 2.98$, $P = 0.126$). There was also a significant time × treatment interaction ($F_{16,72} = 8.24$, $P = 0.006$) for the cumulative number of pupating mealworms as a proportion of the remaining viable larvae, with control mealworms pupating significantly more than JH treated mealworms from day 14 ($P < 0.05$, *post hoc* analysis). Indeed, pupation was entirely inhibited in JH-PL and JH-PH treated mealworms (Figure 1C). There was no time × treatment interaction for the percentage of surviving mealworms ($F_{16,72} = 2.21$, $P = 0.137$), nor any effect of treatment ($F_{2,72} = 0.61$, $P = 0.576$), but there was a significant effect of time ($F_{8,72} = 244.55$, $P < 0.001$), with the proportion of live mealworms gradually decreasing over the 28 days (Figure 1D).

Effects of the juvenile hormone analogue, pyriproxifen, on whole mealworm body composition

Body composition was determined at the end of the study (day 28). Moisture content was significantly increased with JH treatment ($F_{2,9} = 66.70$, $P < 0.001$),

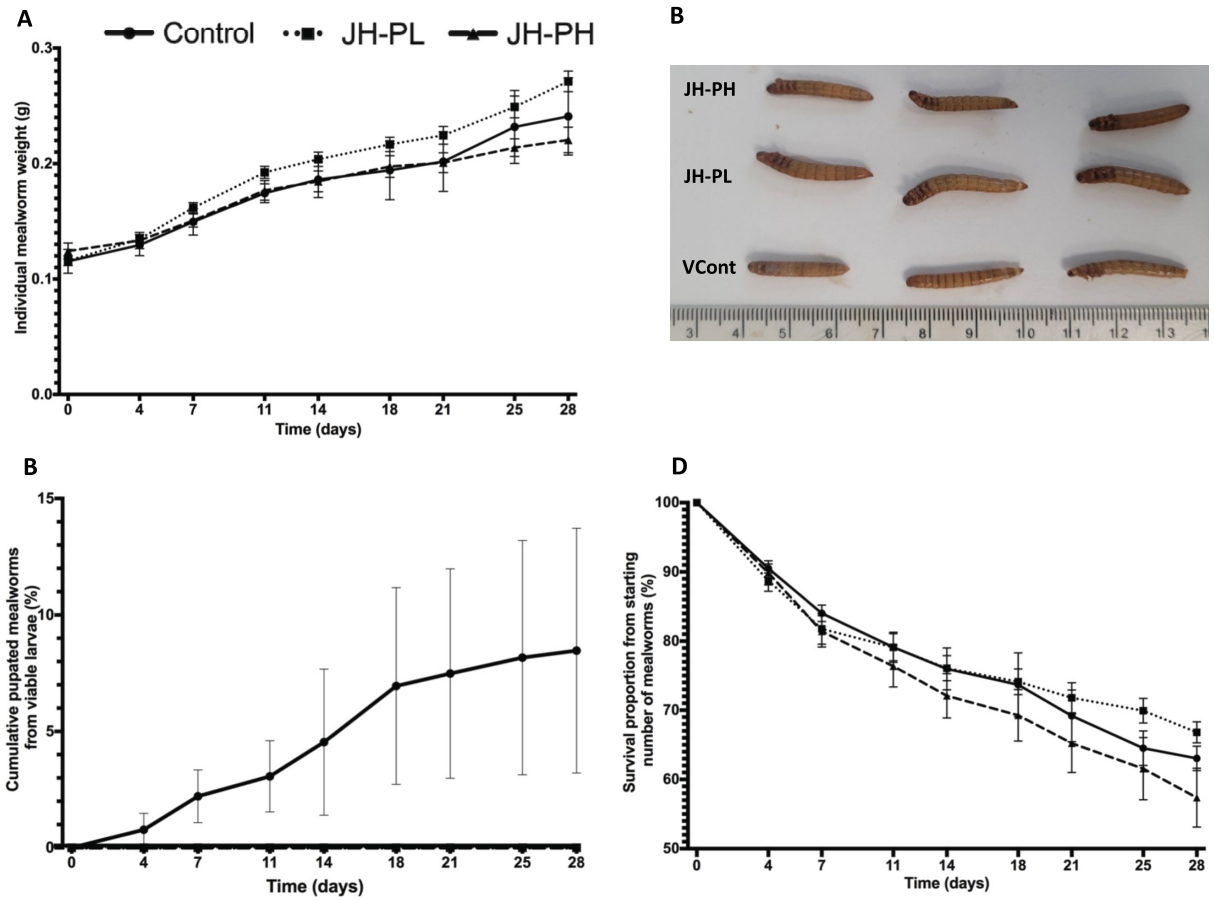

FIGURE 1 Effect of 28 days treatment with the Juvenile Hormone analogue, pyriproxifen, on MW growth characteristics. Control = acetone control, JH-PL = 2 mg pyriproxifen/kg wheat bran, JH-PH = 15 mg pyriproxifen/kg wheat bran. (A) average individual MW weight. There was a trend for treatment × time interaction ($F_{16,72} = 3.81$, $P = 0.052$). There was a significant effect of time ($F_{8,72} = 259.64$, df = 8, $P < 0.001$), but no effect of treatment ($F_{2,72} = 2.98$, df = 2, $P = 0.126$). (B) Images of selected MW from different treatment groups taken at end point of trial (day 28). Scale shown is cm. (C) The cumulative MW pupating as proportion of remaining viable larvae. There was a significant treatment × time interaction ($F_{16,72} = 8.24$, $P = 0.006$). (D) MW survival, indicated by proportion of viable larvae remaining (excluding pupated MW). There was a significant of time $F_{8,72} = 244.55$, df = 8, $P < 0.001$) but no effect of treatment ($F_{2,72} = 0.61$, df = 2, $P = 0.576$), but no interaction. Data are presented as average ± standard error of the mean. Data was analysed by Repeated Measures Analysis (time being the repeated factor) ANOVA.

with both JH-PL and JH-PH being higher than control, and JH-PH having the highest moisture content (Figure 2A). On a dry matter (DM) basis, there was also a significant effect of JH treatment on crude protein content ($F_{2,9} = 262.52$, $P < 0.001$). Both JH-PL and JH-PH had significantly greater protein content than control, with JH-PH having the highest crude protein content at 70.3 g/ 100 g DM, a 46% increase compared to control (Figure 2B). There was also a significant effect of JH treatment on fat content ($F_{2,9} = 888.34$, $P < 0.001$) with lower fat contents in both JH-PL and JH-PH compared to control, with JH-PH having the lowest fat content at 8.9 g/100 g DM, a 68% decrease compared to control (Figure 2C). Consequently, the gross energy content was also lower in both JH-PL and JH-PH compared to control ($F_{2,9} = 460.90$, $P < 0.001$; Figure 2D), with JH-PH again being lowest.

Since there were significant effects of JH treatment on mealworm fat content, the fatty acid composition was also determined and significant differences ($P < 0.001$) were observed for all fatty acids apart from linoleic acid (Table 1). Three saturated fatty acids, myristic, palmitic and stearic acids, were present and all 3 were significantly increased in both JH-PL and JH-PH treatments compared to control, with JH-PH tending to have the highest levels, although for myristic acid there was no significant difference between JH-PH and JH-PL. In contrast, the two monounsaturated fatty acids present, palmitoleic and oleic acids, as well as the essential polyunsaturated fatty acid, α-linolenic acid, were all significantly decreased in both JH-PL and JH-PH treated mealworms compared to control, with JH-PH mealworms having the lowest levels. The most abundant fatty acid present was linoleic acid, making up over

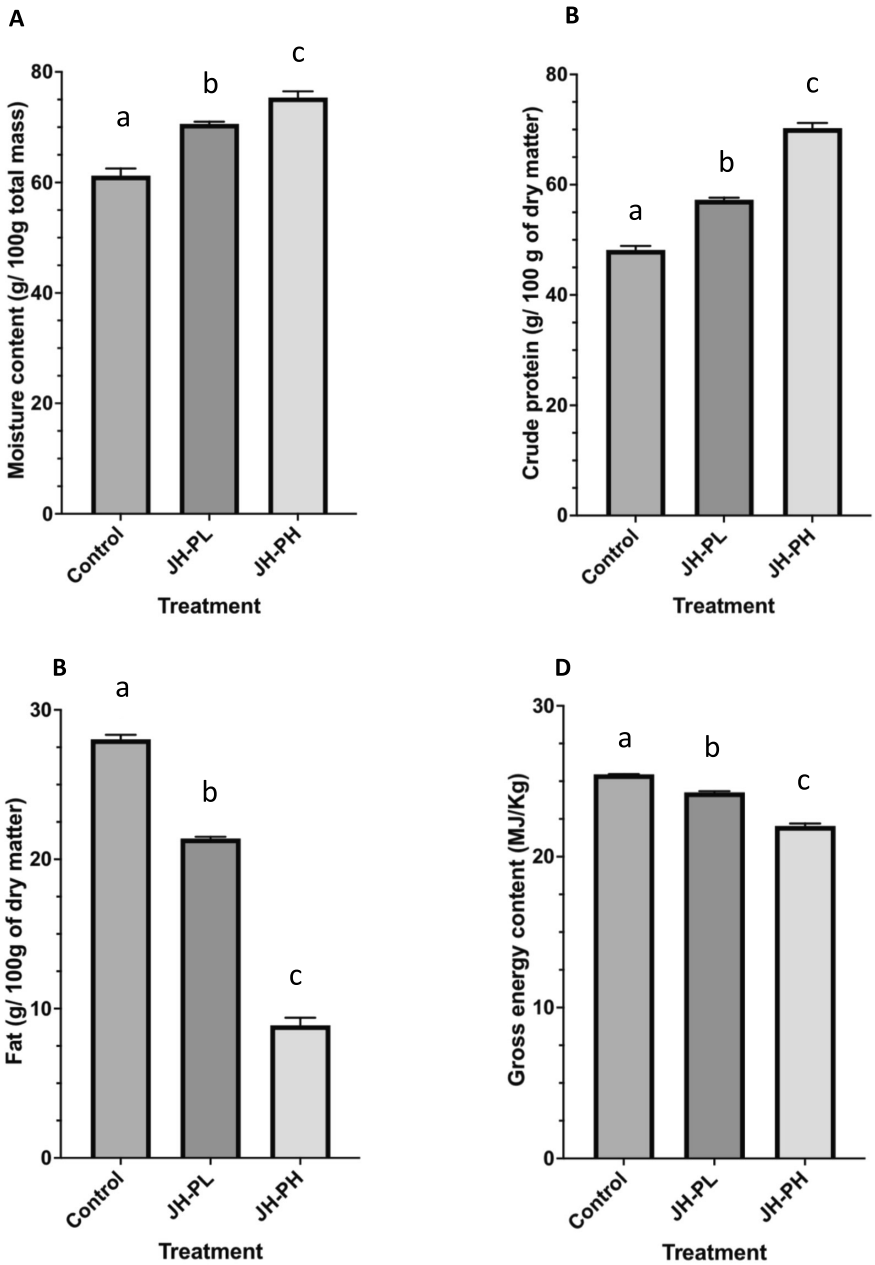

FIGURE 2 The effects of the juvenile hormone analogue, pyriproxifen, on whole MW composition after 28 days treatment. (A) Moisture content (g/100 g DM), (B) crude protein content (g/100 g DM), (C) crude fat content (g/100 g DM), and (D) gross energy content (MJ/kg). There were significant effects of treatment for all measures ($P < 0.001$, one-way ANOVA, with 2,9 degrees of freedom), a,b,c different letters indicate significant differences ($P < 0.05$, Bonferroni host-hoc test). Data are presented as means ± standard error of the mean.

40% of the total fatty acids, but this was not affected by JH treatment, although the levels were numerically higher in the JH-PH group.

As effects of JH were observed for mealworm crude protein contents, the impact on individual amino acids was also determined. Of the essential or indispensable amino acids (IAA) analysed, the branched chain amino acids, Leu and Val, made up the highest proportion of mealworm protein (Table 2). The majority of IAA (His, Ileu, Leu and Thr) were unaffected by treatment, but Lys was significantly increased in the JH-PH compared to control and JH-PL mealworm groups. In contrast, Met and Phe were both significantly decreased in JH-PL treated mealworms compared to both control and JH-PH, whereas Val was significantly decreased in JH-PH compared to both control and JH-PL.

For the non-essential or dispensable amino acids, Asp made up the highest proportion and there were no effects of treatment on Asp, Gly or Ser contents. Arg and Cys were significantly increased in both JH

TABLE 1 The effect of the juvenile hormone analogue pyriproxifen treatment for 28 days on whole mealworm fatty acid composition

Fatty acids[1]	Proportion of fatty acid profile (%)			F-value[2]	*P*-value[3]
	Control	JH-PL	JH-PH		
Myristic (C14:0)	1.87 ± 0.05[a]	2.53 ± 0.08[b]	2.75 ± 0.13[b]	35.99	<0.001
Palmitic (C16:0)	14.57 ± 0.24[a]	15.77 ± 0.10[b]	19.05 ± 0.34[c]	117.44	<0.001
Stearic (C18:0)	1.96 ± 0.04[a]	2.94 ± 0.01[b]	5.78 ± 0.28[c]	165.12	<0.001
Palmitoleic (C16:1)	1.99 ± 0.01[c]	1.61 ± 0.05[b]	1.06 ± 0.03[a]	535.93	<0.001
Oleic (C18:1n9c)	33.95 ± 0.25[c]	31.26 ± 0.58[b]	25.28 ± 0.66[a]	238.16	<0.001
Linoleic (C18:2n6c)	42.57 ± 0.11	43.16 ± 0.67	43.86 ± 0.72	1.91	0.229
α-Linolenic (C18:3n3)	1.81 ± 0.03[c]	1.47 ± 0.07[b]	0.59 ± 0.03[a]	82.46	<0.001

Control = acetone control, JH-PL = 2 mg pyriproxifen/ kg wheat bran, JH-PH = 15 mg pyriproxifen/kg wheat bran.

[1] Individual FAs are expressed as mean % of the total of all fatty acids detected by GCMS ± standard error of the mean (n = 4). Fatty acids with <1% abundance are not shown.

[2] The F-value for One-Way ANOVA of effect of treatment on fatty acid proportion, with 2.9 degrees of freedom for all fatty acids analysed.

[3] *P*-value for One-Way ANOVA of effect of treatment on fatty acid proportion.

[a,b,c] For each fatty acid, means with different superscript letters are significantly different ($P < 0.05$, Bonferroni host-hoc test). Where there are no letters, no significant differences were noted.

TABLE 2 The effect of treatment with the juvenile hormone analogue, pyriproxifen, for 28 days on whole mealworm amino acid composition

	Amino acid[1]	Proportion of amino acid profile (%)			F-value[2]	*P*-value[3]
		Control	JH-PL	JH-PH		
Indispensable amino acids	Histidine	3.18 ± 0.04	2.99 ± 0.04	2.97 ± 0.07	5.72	0.051
	Isoleucine	4.04 ± 0.03	3.98 ± 0.03	4.02 ± 0.15	0.42	0.678
	Leucine	7.12 ± 0.12	7.29 ± 0.19	7.29 ± 0.33	0.2	0.064
	Lysine	4.63 ± 0.09[a]	4.97 ± 0.15[a]	5.44 ± 0.06[b]	36.58	<0.001
	Methionine	1.13 ± 0.02[b]	1.03 ± 0.01[a]	1.10 ± 0.02[ab]	12.07	<0.05
	Phenylalanine	3.80 ± 0.01[b]	3.49 ± 0.04[a]	3.72 ± 0.04[b]	26.53	<0.01
	Threonine	4.52 ± 0.07	4.53 ± 0.06	4.43 ± 0.08	0.93	0.230
	Valine	6.88 ± 0.06[b]	6.93 ± 0.02[b]	6.61 ± 0.13[a]	11.03	<0.05
Dispensable amino acids	Alanine	8.73 ± 0.1	8.84 ± 0.14	8.30 ± 0.09	6.27	<0.05
	Arginine	5.01 ± 0.17	5.40 ± 0.09	5.60 ± 0.07	6.87	<0.05
	Aspartate	14.35 ± 0.08	14.07 ± 0.15	14.54 ± 0.24	2.48	0.178
	Cysteine	0.74 ± 0.01[a]	0.93 ± 0.04[b]	0.93 ± 0.07[b]	10.84	<0.05
	Glutamate	11.99 ± 0.06[b]	11.58 ± 0.05[a]	11.85 ± 0.19[ab]	6.58	<0.05
	Glycine	5.72 ± 0.08	5.70 ± 0.08	5.48 ± 0.15	0.57	0.599
	Proline	7.50 ± 0.01[a]	8.15 ± 0.07[b]	7.59 ± 0.10[a]	34.12	<0.001
	Serine	5.03 ± 0.07	4.86 ± 0.08	4.76 ± 0.03	3.86	0.097
	Tyrosine	5.21 ± 0.07[b]	4.59 ± 0.05[a]	4.49 ± 0.07[a]	40.21	<0.001

Control = acetone control, JH-PL = 2 mg pyriproxifen/ kg wheat bran, JH-PH = 15 mg pyriproxifen/kg wheat bran.

[1] Individual amino acids (excluding tryptophan) are expressed as mean % of the total of all amino acids detected ± standard error of the mean (n = 4), except for JH-PH (n = 3).

[2] The F-value for One-Way ANOVA of effect of treatment on amino acid proportion with 2,8 degrees of freedom for all amino acids analysed.

[3] *P*-values for One-Way ANOVA of effect of treatment on amino acid proportion.

[a,b,c] For each amino acid, means with different superscript letters are significantly different ($P < 0.05$, Bonferroni host-hoc test). Where there are no letters, no significant differences were noted.

treated groups relative to control, whereas Pro was significantly increased in JH-PL compared to control and JH-PH. In contrast, Tyr was significantly reduced in both JH treated groups and Glu was significantly reduced in JH-PL treated mealworms, but JH-PH was not different to control or JH-PL. Ala was reduced in JH-PH compared to control, but JH-PL was not different to either.

SDS-PAGE and Proteomics

Due to differences in protein content, the mealworm soluble protein fractions were analysed by SDS-PAGE to see if there were changes in the protein bands present. The intensity of bands at approximately 150-160 and 45-50 kDa were significantly altered with treatment ($F_{2,8}$ = 25.73, $P < 0.001$ and $F_{2,8}$ = 15.39, $P = 0.004$, respectively), while the distinct protein band at approximately 150-160 kDa visible in the JH-PH treated mealworms, was not visible in the other treatment groups (Figure 3). The band at 45-50 kDa was present in all treatment groups but significantly decreased in the JH-PH treated mealworms compared to control ($P < 0.05$, *post-hoc* analysis). Proteomic analysis of the 150-160 kDa band identified it as vitellogenin (Uniprot accession number Q64EZ1), which is classified as having lipid transporter activity. It was estimated that this band made up approximately 0.9% of all the stained proteins on the SDS-PAGE within the JH-PH treated mealworms (Figure 3). Generally, Vitellogenins are considered egg yolk proteins in insects and other egg laying (oviparous) animals, and are expressed outside the ovaries during the reproductive phase of an insect's lifecycle. It is released in large quantities into the haemolymph and then taken up by oocytes (Tufail *et al.*, 2014).

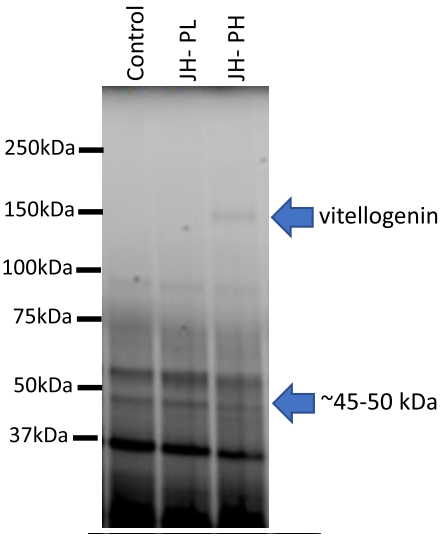

FIGURE 3 The effect of treatment for 28 days with the Juvenile Hormone analogue, pyriproxifen, on whole MW protein composition. 4-15% polyacrylamide gel (Criterion™ TGX Stain-Free™, Bio-Rad, Watford, UK) SDS-PAGE of soluble MW protein (10 ug protein per lane). Molecular weight markers are shown and the arrow indicates the induced expression of protein band at approximately 150-160 and 45-50 kDa. Control = acetone control, JH-PL = 2 mg pyriproxifen/kg wheat bran, JH-PH = 15 mg pyriproxifen/kg wheat bran.

4 Discussion

The response to exogenous JH varies in beetles and moths, but generally, the larvae of these insects undergo additional molts and become giant larvae (Truman, 2019). However, the effects on growth were not as clear for mealworms. Although there was no significant effect of treatment on the growth or the proportion of mealworms surviving, JH-PL treated mealworms tended to be slightly heavier than control and JH-PH mealworms. It appears that the stage of larval development is critical for the response to JH analogues. Application in early instars tends to increase supernumeracy molts, whilst application in the last instar doesn't cause additional molts but has been described as interfering with metamorphosis (Parthasarathy and Palli, 2021), which tends to lead to larger larvae. In holometabolous insects such as *T. molitor* the interaction between the ecdysteroid 20E and JH affects larval-pupal and pupal-adult metamorphosis. The presence of 20E causes larval-larval molting, but if JH is absent it induces larval-pupal as well as pupal-adult molts, therefore JH is involved in reducing the transition of larvae to pupae and adults (Riddiford, 1994). In late instar mealworms, exposure to pyriproxyfen would be expected to keep them in the larval stage. Although we could not determine the instar number of the mealworms used in our study, it is presumably the final, or close to it, as our control mealworms pupated during the course of the experiment. Our results clearly indicate there was inhibition of metamorphorsis, with no mealworms pupating in the pyriproxifen treated groups, whereas 5-10% of larvae pupated in the control group. Pyriproxifen is a well-studied inhibitor of pupation (Dhadialla *et al.*, 1998) that mimics the effects of JH in insects, following similar pathways that involve binding to methoprene-tolerant (Met), a basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) family protein transcription factor. This can form homo and heterodimers with other transcription factors to influence gene transcription by subsequently binding to JH response elements (JHRE) within gene promoters. The zinc-finger transcription factor, *Krüppel-homolog 1*

(*Kr-h1*), is an early JH response gene, which has an anti-metamorphosis effect (Zhang *et al.*, 2022). Pupation was affected for both doses of pyriproxifen relative to the control, but there was no effect on survival. However, it has previously been reported that very high doses of pyriproxifen can cause mortality due to abnormal development. For example, cotton leafworm (*Spodoptera littoralis* Boisduval) larvae treated with 75 mg pyriproxifen/kg resulted in 100% mortality at day 6 of treatment due to a series of physical malformations, such as extrusion of the hindgut, double head capsule formation, loss of haemolymph and failure to shed old cuticle (Nasr *et al.*, 2010).

Previously, it has been reported that the moisture content of mealworms declines as larvae grow and approach pupation (Kröncke *et al.*, 2023). The moisture content of mealworms given the control feed was similar to that previously reported, but that of the pyriproxifen-treated mealworms was significantly higher than that reported in any stage of mealworm development (Kröncke *et al.*, 2023). On a dry matter basis, pyriproxifen treatment was associated with increased protein content and decreased fat content. Obviously, as one component of dry matter content increases the proportion of another component(s) will decrease. The mealworms from the control group were estimated to be 48% protein and 28% fat (on a dry matter basis), which is within the range previous reported (Hawkey *et al.*, 2021). JH-PH induced a substantial repartitioning effect, resulting in an increase in the proportion of protein (from 48% to 70%) and a decrease in fat (from 28% to 8%).The JH stimulated changes were associated with a reduction in the gross energy of the mealworms (on a DM basis). It is likely that the compositional changes are due to a decrease in mealworm fat deposition with pyriproxifen treatment, which is the opposite to what happens during normal mealworm growth, where the dry matter proportion of protein decreases and fat increases (Yu *et al.*, 2021; Kröncke *et al.*, 2023). In comparison, beta-adrenergic agonists, the most potent repartitioning growth agent in livestock, have the greatest effect in cattle reducing fat by 30% (Mersmann, 1998), significantly lower than the effect of pyriproxifen on mealworms. The macronutrient changes in the pyriproxifen treated mealworms could be due to effects on synthesis and/ or degradation of lipid and/or protein (or a combination). Previous research indicates that 20E and JH influence lipid metabolism (Riddiford *et al.*, 2003). Kang *et al.* (2017) described how, in *Drospohila* larvae, the loss of the JH signalling pathway dependent transcription factor, *Kr-h1*, was associated with decreased TAG, which appeared to be mediated by a reduction in lipogenesis and fatty acid synthesis. They suggested that the effects of JH were through the integration of its signalling pathway with insulin signalling (insulin-like peptides) which, like in other animals, is one of the key endocrine factors directly affecting lipid metabolism (stimulating fatty acid synthesis and TAG deposition). These observations appear to contradict the findings of the current study, where JH treatment led to a decrease in fat deposition. However Shpigler *et al.* (2021) demonstrated that in queen bees, *Bombus terrestris* L., JH induced an increase in resting metabolic rate and increased protein turnover, along with an increase in food consumption and reduced lipid levels. The response to exogenous JH varies, with Coleoptera and Lepidoptera tending to undergo additional molts and become larger, whereas Diptera do not (Truman, 2019), indicating fundamental differences in responses between insects. In addition, there are multiple other endocrine factors that also influence lipid metabolism (Toprak *et al.*, 2020), therefore the concentration of other endocrine factors may have influenced the response to exogenous JH observed here at this late stage of *T. molitor* larval development (pre-pupation).

An alternative hypothesis to a decrease in fat deposition could be that protein deposition is increasing. The SDS-PAGE analysis of mealworm whole body proteins did not show dramatic or global changes in protein composition with very few significant changes in protein band intensity, suggesting the types protein being synthesised was not changed. However, the high dose of pyriproxifen resulted in an increase in a specific band identified as vitellogenin, although this was estimated a small proportion (0.9%) of the detected protein. It has long been known that synthesis of vitellogenin is increased by JH analogues (Edwards *et al.*, 1993). Expression of this protein is associated with insect reproduction and it is therefore found in adult insects. Vitellogenin is a phospholipoglycoprotein which is synthesised in the fat body and involved in lipid transport from the fat body to the oocytes, but is then deposited in the yolk body after being converted to vitellin (Wu *et al.*, 2021). Therefore in addition to lipid transport, vitellogenin deposition in oocyes provides a source of protein for development of the embryo. In insects, the synthesis of vitellogenin is regulated by JH and 20E but the role of each varies across insect orders. In holometabolous Coleopteran, like the red flour beetle *Tribolium castaneum* (Herbst), vitellogenin synthesis in the fat body is controlled by JH, whilst 20E effects are

associated with oocytes (Sheng *et al.*, 2011). In sexually mature females, vitellogenin is associated with nutrient deposition in oocytes. However vitellogenin synthesis can also take place in males in some species (Tufail *et al.*, 2014), but in our study we could not distinguish between mealworm sexes, hence we are unable to determine whether there were any sex differences in response. Although vitellogenin synthesis is associated with adult insects it has also found to be expressed in larvae (Guidugli *et al.*, 2005), and we have shown that it is expressed in early *T. molitor* larvae, as well as in the pupae and beetles (unpublished data). This suggests vitellogenin has additional roles to that in reproduction. Indeed, a recent review (Kodrík *et al.*, 2023) suggested vitellogenin could be involved a variety of functions, including immunity and wound healing. However, in our study it is the higher concentration of the pyriproxifen that is stimulating the production of vitellogenin in the mealworms, presumably from their fat bodies. While there is clearly an increase in the amount vitellogenin present, it is clearly not responsible for the overall increase in proportion of protein. While further work is required, we suggest that it is most likely the decrease in lipid deposition that is responsible for the increase in the proportion of protein on a dry matter basis.

In this study, both treated and control mealworms contained higher proportions of unsaturated than saturated fatty acids, and the predominant fatty acids present were oleic and linoleic acids. This agrees with what has been previously reported (Kröncke *et al.*, 2023) and this composition is thought to be a reflection of the fatty acid content of their feed. Linoleic acid is the predominant fatty acid in the wheat bran (which was used as feed in this study) representing over 50% of the total fatty acids present, compared to 15% oleic acid (Feed Tables, 2021a). Pyriproxifen treatment resulted in an increase in proportion of saturated fatty acids and a decrease in monounsaturated fatty acids, with oleic acid decreasing the most. The proportion of the major polyunsaturated fatty acid, linoleic acid did not change. These changes might be due to a decrease in stearoyl-CoA desaturase (SCD) activity, thereby reducing the desaturation of stearic acid to oleic acid (and palmitic acid to palimitoleic acid).

Pyriproxyfen is used as a pesticide and is generally considered to have relatively low-toxicity to humans and animals compared to other synthetic insecticides (Parthasarathy and Palli, 2021). The JH analogues are restricted to pest control applications and there are EFSA-recommended minimal risk levels for pyriproxyfen in food products (EFSA *et al.*, 2022). The treatment of mealworms with pyriproxyfen caused the expected inhibition of pupation. However, this was associated with a repartitioning effect resulting in an increase in the proportion of protein (to 70%) and a decrease in fat (to 8%), This results in a composition that is similar to fish meal (Feed Tables, 2021b) and indicates that mealworm nutrient composition can be manipulated. Restrictions on food contamination and the prohibition of use of pharmaceutical growth promoters in food production livestock would likely prevent the use of JH analogues in insect food production systems. However, further exploration of the mechanisms by which pyrioxyfen effects body composition could identify potential targets for selective breeding and/or genetic manipulation to enhance nutrient composition.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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